

The University of Chicago

I. THE OXIDATION-REDUCTION POTENTIAL
OF CYTOCHROME C REDUCTASE

II. THE EFFECT OF 2,4-DINITRO-*o*-CYCLO-
HEXYL-PHENOL ON THE CYTOCHROME
C OXIDASE SYSTEM

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE PHYSICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY
DECEMBER, 1941

By
BERNARD BLOCK

CHICAGO, ILLINOIS
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I

THE OXIDATION-REDUCTION POTENTIAL OF CYTOCHROME C
REDUCTASE



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INTRODUCTION

Alloxazine-proteid cytochrome c-triphosphopyridine nucleotide or more commonly cytochrome c reductase serves as a very important link in the chain of oxidative enzymes. The answer to two fundamental problems in enzyme chemistry is to a far degree furnished in the discovery of this enzyme. The question of the function of the yellow enzymes and the question of the connection of certain dehydrogenase systems to oxidase systems is herewith largely clarified.

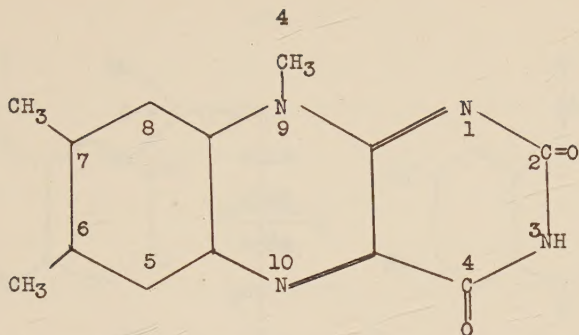
As cytochrome c reductase is a reversible oxidation reduction system, it is necessary that its oxidation-reduction potential be determined for a more complete understanding of its function. The purpose of this investigation was the determination of the oxidation-reduction potential of the enzyme. The approach to this problem was simplified by the great amount of work done in the study of the "old" yellow enzyme and its prosthetic group, lactoflavine phosphate. Cytochrome c reductase having the same prosthetic group as the "old" yellow enzyme should and does have many similar properties. A review of the investigations on the "old" yellow enzyme and particularly its prosthetic group is very helpful in the understanding of the characteristics of the cytochrome c reductase.

The earliest reference to natural lactoflavine was provided by Blyth in 1879 who described a yellow pigment, lactochrome, which he obtained from milk. The study of lactochrome was again resumed in 1925. In 1932 and in 1933 a number of workers isolated a yellow pigment which they suspected of having enzymatic properties (1,2). Warburg and Christian in 1932 isolated and purified

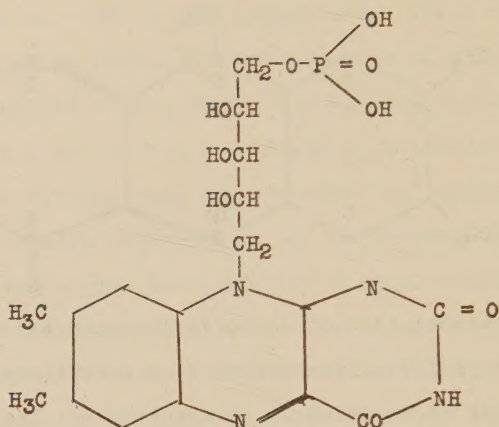
what is now known as "old" yellow enzyme (3). These workers showed that the yellow enzyme had the property of oxidizing reduced triphosphopyridine nucleotide (T.P.N.) and diphosphopyridine nucleotide (D.P.N.) and of being oxidized by molecular oxygen.

Extensive studies were made on both the chemical constitution and the enzymatic function of yellow enzyme. Warburg and Christian found the enzyme to consist of a small molecular weight fraction of relatively high heat stability in combination with a high molecular weight fraction of low heat stability. The properties and chemical analysis of the large fraction indicated a protein, consequently the small fraction in combination with it could be considered a prosthetic group. The combined compound exhibited the property of reversible dissociation. The dissociation constant of the reduced enzyme is very small, 1×10^{-9} (4), indicating a tightly bound complex. It is likely that the dissociation constant of the oxidized form is even smaller although it has as yet not been determined. On dissociation it was found that the yellow color of the compound was due to the low molecular weight fraction or the prosthetic group.

Warburg and Christian found that irradiation of the dissociated yellow pigment in alkaline solution yielded a photo derivative that could be crystallized (5). Irradiation of pure lactoflavine yields a photo derivative, identical with that of Warburg. The empirical formula of pure lactoflavine, $C_{17}H_{20}N_4O_6$, and that of the photo derivative, $C_{12}H_{13}N_4O_2$, indicated the splitting off of a $C_4H_7O_4$ group, possibly carbohydrate. After extensive investigation of the properties of the photo derivative, lumiflavine, Kuhn and his co-workers determined the structure by synthesis in 1934 (6). They found it to be 6,7,9,trimethylalloxazine.



The association of lactoflavine with the prosthetic group of Warburg's yellow enzyme having been established, the nature of the side chain dissociated by irradiation was the next subject for extensive research. Simultaneously Kuhn (7) and Karrer (8) synthesized a compound identical with lactoflavine by introducing a side chain of d-ribose in the nine position of the lumiflavine. The synthetic product exhibited complete vitamin potency as did Warburg's prosthetic group. In 1934, Theorell (9) demonstrated the differing electrophoretic characters of lactoflavine and the yellow enzyme prosthetic group which was found to be lactoflavine phosphate. Also he was unable to resynthesize the yellow enzyme from free protein and lactoflavine but was able to do so with lactoflavine phosphate. This established the structure of the prosthetic group except for the position of the phosphate substituent. Further work by Karrer (10) and others indicated the position of the phosphate as on the five carbon atom of the side chain. The complete picture of the structure of lactoflavinephosphate or the yellow enzyme prosthetic group as now accepted is that of the alloxazine-mononucleotide.

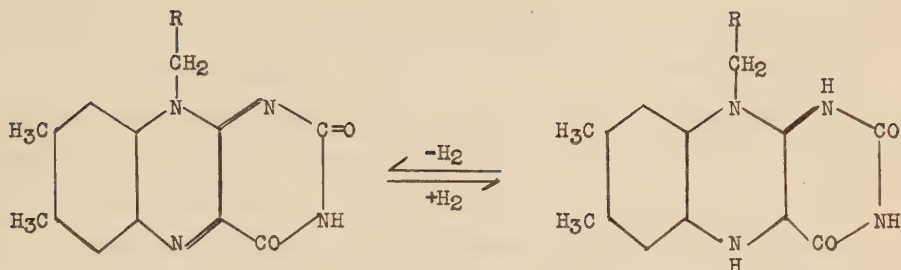


The exact nature and the position of the binding between the protein and the prosthetic group is yet unknown although Kuhn and Boulanger (11) give evidence for the phosphate group and the nitrogen in the three position for the points of binding in the lactoflavine phosphate.

The absorption spectrum of the yellow enzyme (Fig. 1) (12) shows peaks at 275, 380, and 465 mμ. The spectrum of the lactoflavine (Fig. 2) is very similar though shifted farther toward the blue with peaks at 270, 365, and 445 mμ (13).

Kuhn and Boulanger (11) studied the oxidation-reduction potentials of both the lactoflavine phosphate and the yellow enzyme potentiometrically. Their results placed the potential of the prosthetic group alone at pH 7.0 between -0.185 and -0.191v. For the yellow enzyme they reported a much higher potential. E'_0 at pH 7.0 was found to be between -0.059 and -0.066v.

The point of attack in the reversible oxidation of the lactoflavine, free or combined, has been determined by Theorell (14). This can best be shown as below.



The yellow enzyme has been found widely in yeasts and bacteria and lactoflavine alone can be prepared from animal tissue, mainly liver. Warburg and others have demonstrated the ability of the yellow enzyme to oxidize reduced T.P.N. and to a lesser degree D.P.N. and to reduce oxygen. However, the great dependence of the respiratory system on the two nucleotides and the slow rate of reaction between oxygen and yellow enzyme negated this as a respiratory path. In addition, the entire respiration of yeast had been found to go through the cytochrome c-cytochrome c oxidase iron bearing systems to which no link had been found for the yellow enzyme. Thus a situation existed in which a widely occurring and apparently important enzyme could not be shown to function in physiological respiration.

In order to show the mode of oxidation of the nucleotides as well as find the function of the yellow enzyme, it was necessary to find the connection between the lactoflavine bearing protein and the cytochrome c-cytochrome c oxidase system. This has been accomplished with the discovery of cytochrome c reductase.

CYTOCHROME C REDUCTASE

The first evidence of a reaction between the yellow enzyme and cytochrome c was reported by Theorell in 1936 (15). The reaction rate here was too slow to be of any physiological significance. In 1938, Haas detected an enzyme that oxidized reduced

T.P.N. and was itself oxidized by cytochrome c (16). Subsequently working with Hogness and Horecker, he has isolated and purified this material (4). The cytochrome c reducing enzyme has been shown to be a flavine proteid employing the alloxazine mononucleotide as a prosthetic group and a new protein as a carrier. According to the Warburg system of nomenclature (7), the enzyme would be termed:

alloxazine-proteid_{cytochrome c-triphosphopyridine nucleotide}.
For brevity the name commonly applied is cytochrome c reductase.

The Warburg designation for the enzyme serves to indicate not only the structure of the proteid but also the role played by it in the oxidation scheme. Here the subscript following the name shows that cytochrome c acts as an oxidizing agent and T.P.N. as a reducing agent for the enzyme.

The reaction employed as a test for the reductase can serve as a model for its behavior. A mono-phosphorylated sugar, hexosemono phosphate, reacts with T.P.N. giving up two hydrogen atoms to the coenzyme. This reaction takes place only in the presence of the protein carrier "zwischenferment." The T.P.N. is then oxidized by the flavine of the reductase, again only in the presence of a carrier protein though this time that of the flavine proteid. The zwischenferment is not essential for the second reaction. The reduced flavine, still in combination with its protein, is then oxidized by cytochrome c. For purposes of the test the cytochrome c is retained in the reduced state. In the cell, on the other hand, the cytochrome c is immediately oxidized by a complex of oxidizing enzymes, cytochrome c oxidase, which are not too well known at present and which have not as yet been isolated. Cytochrome c reductase will react directly with oxygen, but even at atmospheric pressure the rate of reaction is very slow. Slower

even than that of the "old" yellow enzyme.

The reactions above take place with the formation of dissociable complexes. The rate of the reactions and the dissociation constants of the complexes have been studied by Haas, Harrer and Hogness and will shortly appear in a report by these workers.

The enzyme, as characteristic of most proteids, is a labile compound, very easily destroyed by heat, some organic solvents and wide variations of pH. It is most stable in the presence of high ammoniumsulphate concentrations at pH 9 in solution or when dried as the ammoniumsulphate precipitate which has been rendered alkaline. A temperature of 50° C. or higher will completely destroy enzymatic activity in ten minutes. At lower temperatures activity is destroyed less rapidly and also differentially. Standing at room temperature for a period of a day or more will cause almost complete loss of activity toward cytochrome c while the activity toward oxygen will be affected only slightly (18). Haas has also found that in water solution the very highly purified enzyme exhibits greater stability than does the less pure preparation. At pH of less than 4.5 the enzyme is rapidly destroyed and precipitated. Acetone and dioxane will irreversibly denature the protein and destroy enzyme activity.

The loss of enzymatic activity above is associated in all cases with the destruction of the protein and simultaneous dissociation of the prosthetic group.

The absorption spectrum of the reductase (Fig. 3) resembles closely that of the "old" yellow enzyme of Warburg. There is a slight shift in absorption maxima. The peaks of the reductase lie at 275, 385, and 455 mμ.

The preparation of the cytochrome reductase from yeast illustrates well the techniques of fractional precipitation and

adsorption on inert gels used in protein purification. As presented here the preparation embodies a few changes from that originally published by Haas, Horecker and Hogness (4).

PREPARATION

Source Material.--Top ale yeast of the strain Saccharomyces cerevisiae I, Hanson, obtained from Drewry's, Ltd., was washed at 0° with tap water and then subjected to high pressure to remove the excess water. By blowing air over a thin layer of the yeast for a few hours, it was thoroughly dried. The dried yeast can be stored at room temperature without apparent loss of activity.

Step 1, Extraction of Enzyme by Autolysis.--4 kilos of the dry yeast are suspended in 14 liters of water and kept for 33 hours at 20°. The suspension is centrifuged and 6.9 liters of clear solution are obtained which contain most of the enzyme. The residue is washed with 6.5 liters of water and centrifuged. The resulting supernatant solution is combined with the original to give 13.8 liters of an enzyme solution which contained 1400 gm. of dry substance. The purity of the enzyme in the first extract is 0.0017. The enzyme is very easily denatured and the whole of the following procedure must therefore be carried out at 0°.

Step 2, Fractionation with Ammonium Sulfate.--At pH 4.5 the enzyme is soluble when the solution is 31 per cent saturated with ammonium sulfate. Under these conditions a large amount of inert proteins is denatured and can be removed. The enzyme is precipitated when the solution is made 51 per cent saturated with respect to ammonium sulfate.

Specifically, 13.8 liters of enzyme solution are cooled to 0°. While the solution is being stirred, 4.2 kilos of solid ammonium sulfate and then 220 cc. of 10 N acetic acid are added.

The saturation is then 51 per cent with respect to ammonium sulfate, pH 4.5. The precipitate is separated by centrifugation. It contains the enzyme and can be kept in this form overnight. The precipitate, which has a volume of 1.1 liters, is suspended in 0.7 liter of water and 3 liters of a solution 31 per cent saturated with ammonium sulfate are added. The insoluble material is separated by centrifugation and discarded.

From the supernatant solution (4 liters) the enzyme is precipitated by adding 470 gm. of ammonium sulfate; the degree of saturation is then 51 per cent. After separation by centrifugation the enzyme is dissolved in 500 cc. of water. The solution contains 87 gm. of dry substance, other than ammonium sulfate. Purity after step (2) = 0.012; purification 7-fold; yield 44 per cent.

Step 3, Dialysis.--For the following precipitation with alcohol it is necessary to remove the dissolved ammonium sulfate. Consequently the enzyme is dialyzed in cellophane tubes against running distilled water for 17 hours at 0°. After the removal of much inactive precipitate by centrifugation, 1200 cc. of enzyme solution containing 52 gm. of protein are left. Purity = 0.0126; purification 1.06-fold; yield 63 per cent.

Step 4, Precipitation with Ethanol.--1200 cc. of dialyzed enzyme solution are adjusted to pH 4.65 by adding 1.5 cc. of 2 N KOH. Slowly and with stirring in an ice bath, 600 cc. of 30 per cent cold ethanol are added. A fine white precipitate is formed which contains the enzyme. After 45 minutes the suspension is centrifuged and the precipitate is dried as rapidly as possible in vacuo over CaCl_2 at 0°. After this step 5.0 gm. of a dry powder are obtained. Purity = 0.076; purification 6-fold; yield 58 per cent.

Step 5, Adsorption on Aluminum Hydroxide Gel; Elution with Alkaline Ammonium Sulfate.--5.0 gm. of the enzyme powder are dissolved in 200 cc. of water. By adding 5 cc. of N KOH the pH is adjusted to about 9. Aluminum hydroxide gel prepared according to Willstatter and Kraut (19) is added fractionally until the supernatant solution is just colorless. Excess adsorbent should be avoided. About 7.0 gm. of aluminum hydroxide are usually necessary to adsorb all the enzyme.

The enzyme is eluted from the aluminum hydroxide by adding 50 cc. of a solution which is 64 per cent saturated with ammonium sulfate and is 0.1 N with respect to NH_4OH . This elution with alkaline ammonium sulfate is repeated twice. From the combined eluates (150 cc.) the enzyme is precipitated by adding 18 cc. of 2 N acetate buffer, pH 4.5, and 30 gm. of solid ammonium sulfate (degree of saturation, 70 per cent). The precipitate is centrifuged for 1 hour at 3000 times gravity to remove as much as possible of the ammonium sulfate solution which interferes in the following step. The enzyme is dissolved in 100 cc. of water and 4 cc. of N NH_4OH are added to adjust the pH to about 9. The solution now contains 360 mg. of protein. $W = 51$; purity = 0.32; purification 4.2-fold; yield 30 per cent.

Step 6, Adsorption on Calcium Phosphate; Elution with Phosphate.--Tricalcium phosphate gel was prepared by following the direction of Utkin (20). The tricalcium phosphate is added to the enzyme solution in small portions until the supernatant solution remains colorless. Approximately 15 gms. of calcium phosphate are sufficient for adsorption of the enzyme. The tricalcium phosphate with the enzyme is washed with distilled water, and the enzyme is eluted with 300 cc. of 0.2 M PO_4 buffer, pH 6.1. The elution is repeated two times with 300 cc. of eluent and once with 200 cc.

and the eluates are combined. The 1100 cc. of the phosphate solution are treated with 495 gms. of solid ammonium sulphate (saturation 0.72). The precipitate is centrifuged for 1 hour at 3000 times gravity. The enzyme is dissolved in 100 cc. of water and 2 cc. of N NH_4OH are added to adjust the pH to about 9. The 100 cc. of solution contain 115 mg. of protein. $W = 87$; purity = 0.54; purification 1.7-fold; yield 48 per cent.

Step 7, Adsorption on Aluminum Hydroxide Gel Repeated.--

100 cc. of the enzyme solution are stirred for 10 minutes with 2.3 gm. of aluminum hydroxide and centrifuged. This treatment is repeated two times with the supernatant solution removing all the color. The precipitates are combined and the enzyme is eluted with 100 cc. of alkaline ammonium sulphate as in step (5). The elution is repeated once with 100 cc. of eluent and once with 60 cc. to remove all color. From the combined eluates (360 cc.), the enzyme is precipitated by adding 29 cc. of 2 N acetate buffer, pH 4.5 and 27 gm. of solid ammonium sulphate (degree of saturation, 70 per cent). The precipitated enzyme is separated by centrifugation. To the precipitate is added 2 cc. of N NH_4OH to adjust the pH to about 9 and the enzyme is dried rapidly with freezing in a vacuum desiccator at 0°C . over P_2O_5 and KOH. The dried precipitate contains 51 mgm. of protein. $W = 160$; purity 1.0; purification 1.9-fold; yield 92 per cent.

The enzyme may be stored over P_2O_5 and KOH in the cold.

The pure enzyme was not used in these experiments. A preparation of about 25 per cent purity was used.

METHOD

Electrometric titrations have been widely used in biological work for the determination of oxidation-reduction potentials. Colorimetric methods of determination have been less commonly used

but offer many advantages and have been increasing in application. The work of Quastel and Whetham (21) and of Thunberg (22) in determining the potential of the succinate-fumarate system and the work of Stotz, Sidwell, and Hogness (23) in determining the potential of cytochrome c are outstanding examples of the effectiveness of this technique.

Essentially the method employs the use of a known reversible oxidation-reduction dye system in equilibrium with the system to be measured. The determination, colorimetrically, of the concentration of the dye and the unknown systems in the oxidized and reduced forms establishes the value of the equilibrium constant. From this value and the known value of the dye potential, the unknown potential may be calculated. The spectral characteristics of the oxidized and reduced forms of the dye and of the unknown system must vary sufficiently from each to allow accurate determination. In cases of stoichiometric reactions, it is possible to obtain values for the equilibrium constant by observing the change of only one of the components.

The per cent of oxidation or reduction and thus the concentration of the oxidized and reduced forms of a component can be determined by comparing an observed change in absorption at a characteristic wavelength with that due to complete change from oxidized to reduced state. Since the equilibrium constant is dependent on the ratio of the oxidized to reduced form it is not even necessary that the concentration be known if the maximum for the concentration involved is first determined.

Calculation of potential from equilibrium constant is accomplished by means of simple well known thermodynamic relationships. The potential of a reversible oxidizing-reducing compound A is given by the equation

$$E_h = E_0 - \frac{RT}{nF} \ln \frac{(A_{red})}{(A_{ox})}$$

where E_h is the observed value, E_o the potential at concentration, $A_{red} = A_{ox}$ at the pH of the medium, and R, T, and F have their familiar value.

In a reaction of A with B, another reversibly oxidizing-reducing substance, the equation may be written



The equilibrium constant is given by

$$K_{eq} = \frac{(A_{red})(B_{ox})}{(A_{ox})(B_{red})}$$

At equilibrium, A and B will have the same E_h value and the equations for their potentials may be combined to give

$$E_{oA} - \frac{RT}{nF} \ln \frac{(A_{red})}{(A_{ox})} = E_{oB} - \frac{RT}{nF} \ln \frac{(B_{red})}{(B_{ox})}$$

or

$$E_{oA} - E_{oB} = \frac{RT}{nF} \ln K_{eq}$$

E_{oB} will then be given by

$$E_{oB} = E_{oA} - \frac{RT}{nF} \ln K_{eq}$$

Thus if E_{oA} is known and K_{eq} determined, E_{oB} may be calculated.

It is evident that the value of E_{oB} is almost independent of small changes in the value of K_{eq} at 25° C. in the case of a two electron change. This low degree of sensitivity decreases the necessary precision in the determination of the equilibrium constant and thus creates a distinct advantage in the technique.

EXPERIMENTAL

Reduction of the enzyme.---For the purposes of this problem, it was necessary to establish conditions for the slow reduction of the cytochrome c reductase. In accord with this, it was essential that the dye used as a standard come to equilibrium very rapidly with the enzyme. In this way the equilibrium established

would be disturbed only to a small degree during the course of the observation.

A reducing system consisting of an excess of hexosemonophosphate, an excess of "zwischenferment" and a limited concentration of T.P.N. was employed. The rate of reduction of the cytochrome c reductase was dependent on the concentration of the T.P.N. and so it was possible to adjust the rate by varying the concentration of the coenzyme. The reduction could readily be followed by observing the change in the absorption at 455 mu.

The conditions most suitable were as follows:

1×10^{-5} moles hexosemonophosphate

5×10^{-11} moles "zwischenferment"

4×10^{-9} moles T.P.N.

5×10^{-8} moles Cytochrome c reductase (approximate)

All dissolved in 3.5 ml. of 0.025 M phosphate buffer of pH 7.0.

Before the addition of the hexosemonophosphate, the reaction mixture with all other components was observed in the spectrophotometer at 455 mu to determine the absorption due to oxidized cytochrome c reductase. The sample for the observation was returned to the original solution which was then placed in an apparatus wherein the oxygen was removed by evacuation. The anaerobic solution was then passed anerobically into the cell in which both the reduction of the enzyme and the reaction with the dye took place. Only 3 ml. (the capacity of the cell) of the reaction mixture were transferred and used in the experiment.

Dye.--Assuming that the potential of the cytochrome c reductase were similar to that of the "old" yellow enzyme, the dye most likely to establish an equilibrium with the reductase with a value of the equilibrium constant approaching one would be indigo

tetra sulphonate. The potential of the indigo tetra sulphonate at the pH of the experiments is given by Clark (24) as $-0.045v$. Preliminary experiments indicated that this dye, on addition to reduced cytochrome c reductase in approximately equimolar quantities, was only partially reduced and only partially oxidized the reduced enzyme. It was also apparent that the equilibrium was established rapidly and changed but slowly during the observation.

The visible absorption spectra (Fig. 3) of the oxidized and the reduced forms of the dye were obtained and a maximum observed at 590 m μ for the oxidized dye. Reduction removed completely the absorption at 590 m μ but created an absorption band increasing toward the violet region and including, though to a small degree, the wave length 455 m μ at which the enzyme was observed. The change in absorption due to the dye at 455 m μ necessitated a correction in the determination of the enzyme change.

To ascertain whether the dye was reduced by the reducing system alone with no cytochrome c reductase present, an experiment was conducted in which the dye was present in a cell in which all components except the reductase were present. No reduction of the dye was observed.

Before addition to the reduced enzyme solution in the absorption cell, the dye solution was treated for removal of oxygen. A stream of nitrogen, from which traces of oxygen were removed, was passed through the solution for an hour. The concentration of oxygen was reduced thus so that a test addition of water, similarly treated, equal in volume to the dye solution to be added but containing no dye, produced no change in the enzyme absorption other than that caused by dilution. In addition it was evident that if the readily autoxidizable dye was not immediately reoxidized after being reduced, as was found, the concentration of the

oxygen must have been at a negligibly low level.

The anaerobic dye was introduced into the cell containing the reduced enzyme by means of a fine pipette. Due to the increase in volume, the solution rose in the cell neck blocking off the contact with the air. The solution in the neck which was in contact with the air was ejected through the capillary in the cell stopper when the stopper was reinserted. A small volume of solution remained in the capillary and protected the main portion of the solution from air. The effectiveness of this technique was proven by the addition of anaerobic water and also by the maintenance of reduced dye in solution as before.

The spectrophotometer used for these experiments consisted of a monochromater, a photo cell, amplifying circuit and galvanometer. The temperature of the chamber in which the cell was placed for observation was regulated for constancy. The deflection of the galvanometer was proportional to the light transmitted.

In operation, the cell in which the reaction was to be carried out was compared with a standard cell using water in each. The correction due to variation in cells was then applied to the observations made under similar conditions.

During the observation, the total light striking the reaction cell was given by the corrected value of the galvanometer deflection, I_0 , produced by the light transmitted by the standard cell. The absorption ratio was then I_0 divided by the galvanometer deflection I caused by the light transmitted by the reaction cell. Assuming Beers' Law valid, the concentration of the reactant was proportional to $\log I_0/I$. As mentioned previously, it was not necessary to know the value of the concentration. The change of the value of $\log I_0/I$ of the completely reduced enzyme to that of the equilibrium concentration compared with the total

possible change gave the per cent oxidation and thus the ratio of oxidized to reduced enzyme. The same was true of the dye.

As the experiment was performed, after complete reduction of the cytochrome c reductase, the dye was introduced and the absorption at 590 μ determined immediately. The wave length was then changed to 455 μ and the absorption in this region determined within one minute. The change due to continued reduction was negligible.

In the experiments that follow, numbers II and III represent two different concentrations of dye added to the same enzyme solution in a very short time interval.

Experiment I

T 25° C. Cell 10.00 mm. pH 7.00 (0.025 M PO_4)

Preliminary determination of maximum change of absorption.

1. 0.10 ml indigo tetra sulphonate (4.44×10^{-8} moles) to buffered at pH 7.00 (0.025 M PO_4).

	Wave Length (μ)	I	I_0	Log I_0/I
Oxidized	590	267	480	0.255
Reduced	590	461	480	0.017
Oxidized	455	464	480	0.015
Reduced	455	395	480	0.085

Maximum change of log I_0/I at 590 μ = 0.238

Maximum change of log I_0/I at 455 μ = 0.070

2. Enzyme and reducing system.

	Wave Length (μ)	I	I_0	$\log I_0/I$
Oxidized	455	340	480	0.150
Reduced	455	391	480	0.089

Maximum change of $\log I_0/I$ at 455 μ = 0.061

Addition of dye to reduced enzyme.

	Wave Length (μ)	I	I_0	$\log I_0/I$
Before addition	590	439	480	0.038
Before addition	455	391	480	0.089
After addition	590	269	480	0.251
After addition	455	345	480	0.143

Change of $\log I_0/I$ at 590 μ = 0.213

Change of $\log I_0/I$ at 455 μ = 0.054

Experiment II

T 25° C. Cell 10.00 mm. pH 7.00 (0.025 M PO_4)

Preliminary determination of maximum change of absorption.

- 0.05 ml. Indigo tetra sulphonate (2.22×10^{-8} moles) to
3.00 ml. buffered at pH 7.00 (0.025 M PO_4).

	Wave Length (μ)	I	I_0	$\log I_0/I$
Oxidized	590	357	480	0.128
Reduced	590	470	480	0.009
Oxidized	455	472	480	0.007
Reduced	455	436	480	0.042

Maximum change of $\log I_0/I$ at 590 μ = 0.119

Maximum change of $\log I_0/I$ at 455 μ = 0.035

2. Enzyme and reducing system.

	Wave Length (μ)	I	I ₀	Log I ₀ /I
Oxidized	455	300	480	0.204
Reduced	455	360	480	0.125

Maximum change of $\log I_0/I$ at 455 μ = 0.079

Addition of dye to reduced enzyme.

	Wave Length (μ)	I	I ₀	Log I ₀ /I
Before addition	590	425	480	0.052
Before addition	455	360	480	0.125
After addition	590	366	480	0.118
After addition	455	310	480	0.190

Change of $\log I_0/I$ at 590 μ = 0.066

Change of $\log I_0/I$ at 455 μ = 0.065

Experiment III

T 25° C. Cell 10.00 mm. pH 7.00 (0.025 M PO₄)

Preliminary determination of maximum change of absorption.

- 0.10 ml. Indigo tetra sulphonate (4.44×10^{-8} moles) to 3.00 ml. buffered at pH 7.00 (0.025 M PO₄).

	Wave Length (μ)	I	I ₀	Log I ₀ /I
Oxidized	590	267	480	0.255
Reduced	590	461	480	0.017
Oxidized	455	464	480	0.015
Reduced	455	395	480	0.085

Maximum change of $\log I_0/I$ at 590 μ = 0.238

Maximum change of $\log I_0/I$ at 455 μ = 0.070

2. Enzyme and reducing system.

	Wave Length (μ)	I	I_0	$\log I_0/I$
Oxidized	455	300	480	0.204
Reduced	455	360	480	0.125

Maximum change of $\log I_0/I$ at 455 μ = 0.079

Addition of dye to reduced enzyme.

	Wave Length (μ)	I	I_0	$\log I_0/I$
Before addition	590	425	480	0.052
Before addition	455	360	480	0.125
After addition	590	281	480	0.232
After addition	455	304	480	0.198

Change of $\log I_0/I$ at 590 μ = 0.180

Change of $\log I_0/I$ at 455 μ = 0.073

EQUILIBRIUM CONSTANTS

Experiment I

Maximum change of $\log I_0/I$ at 590 μ due to dye	= 0.238
Observed change of $\log I_0/I$ at 590 μ	= 0.213
Per cent of total dye in oxidized state	= 89.5%
Per cent of total dye in reduced state	= 10.5%
Ration $\text{dye}_{\text{red}}/\text{dye}_{\text{ox}}$	= 0.117
Maximum change of $\log I_0/I$ at 455 μ due to enzyme	= 0.061
Observed change of $\log I_0/I$ at 455 μ	= 0.054
Change of $\log I_0/I$ at 455 μ due to 10.5% reduced dye	= 0.007
Change of $\log I_0/I$ at 455 μ due to residual dye absorption	= 0.015
Corrected change of $\log I_0/I$ at 455 μ due to enzyme	= 0.039

Per cent of total enzyme in oxidized state	= 64.0%
Per cent of total enzyme in reduced state	= 36.0%
Ratio enzyme _{ox} /enzyme _{red}	= 1.78
Equilibrium constant = (Ratio dye _{red} /dye _{ox}) x (Ratio enz _{ox} /enz _{red})	
Equilibrium constant = 0.208	

Experiment II

Maximum change of log I ₀ /I at 590 mμ due to dye	= 0.119
Observed change of log I ₀ /I at 590 mμ	= 0.066
Per cent of total dye in oxidized state	= 55.5%
Per cent of total dye in reduced state	= 44.5%
Ratio dye _{red} /dye _{ox}	= 0.802
Maximum change of log I ₀ /I at 455 mμ due to enzyme	= 0.079
Observed change of log I ₀ /I at 455 mμ	= 0.065
Change of log I ₀ /I due to reduced dye	= 0.016
Change of log I ₀ /I due to residual dye absorption	= 0.007
Corrected change of log I ₀ /I at 455 mμ due to enzyme	= 0.042
Per cent of total enzyme in oxidized state	= 53.2%
Per cent of total enzyme in reduced state	= 46.8%
Ratio enzyme _{ox} /enzyme _{red}	= 1.14
Equilibrium constant = 0.910	

Experiment III

Maximum change of log I ₀ /I at 590 mμ due to dye	= 0.238
Observed change of log I ₀ /I at 590 mμ	= 0.180
Per cent total dye in oxidized state	= 75.7%
Per cent of total dye in reduced state	= 24.3%
Ratio dye _{red} /dye _{ox}	= 0.321
Maximum change of log I ₀ /I at 455 mμ due to enzyme	= 0.079
Observed change of log I ₀ /I at 455 mμ	= 0.073
Change of log I ₀ /I at 455 mμ due to reduced dye	= 0.017

Change of $\log I_0/I$ due to residual dye absorption	= 0.015
Corrected change of $\log I_0/I$ at 455 μ due to enzyme	= 0.041
Per cent total enzyme in oxidized state	= 51.9%
Per cent of total enzyme in reduced state	= 48.1%
Ratio enzyme _{ox} /enzyme _{red}	= 0.927
Equilibrium constant = 0.299	

Enzyme potential.--The reaction between indigo tetra sulphonate and the cytochrome c reductase may be expressed by



The equilibrium constant for the reaction is given by

$$K_{\text{eq}} = \frac{(\text{Dye}_{\text{red}}) \times (\text{Cyto Red}_{\text{ox}})}{(\text{Dye}_{\text{ox}}) \times (\text{Cyto Red}_{\text{red}})}$$

E_0' , the potential at pH 7, for the Cytochrome reductase may be calculated from the following equation:

$$E_0' = E_{01}' - \frac{RT}{nF} \ln K_{\text{eq}}$$

where E_{01}' is the potential of the indigo tetra sulphonate. For these conditions, the value is -0.045 v.

Using the values of K_{eq} obtained in experiments I, II, and III, the values for the potentials are found to be as follows:

Experiment	K_{eq}	E_0'
I	0.208	-0.025
II	0.910	-0.044
III	0.299	-0.029

The potential of cytochrome c reductase apparently lies in the neighborhood of -0.030 v.

CONCLUSION

Although the results of experiments I and III agree quite closely, the divergence of Experiment II and of many other experiments places these results in question. It is possible that the difficulty in obtaining consistent results lay in the dye system employed. This can be verified by the use of another dye system of similar potential. It may also be that this technique for determining potential will give accurate and consistent results in the case of a stable enzyme such as cytochrome c and yet be less reliable in the case of a labile enzyme as the reductase. This consideration would be influenced by the dye system used in the experiment.

Before any conclusions be drawn from the value of the potential of the cytochrome c reductase obtained here, it would be advisable to determine the potential with another dye system.

SUMMARY

1. The method for colorimetric determination of the oxidation-reduction potential of a reversible oxidation-reduction system was discussed.
2. Values for the potential of the cytochrome c reductase system were obtained.
3. The values obtained should be confirmed by the use of another dye system.

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II

THE EFFECT OF 2,4-DINITRO-*o*-CYCLOHEXYL-PHENOL ON THE CYTOCHROME C OXIDASE SYSTEM

INTRODUCTION

In a series of experiments carried on since 1935, Krah1, Clowes and co-workers (1,2) have studied the effect of substituted phenols on the respiration and cell division of *Arbacia* eggs. The effects observed consisted of a reversible stimulation of oxygen consumption at low phenol concentrations and then at higher phenol concentrations a slight inhibition of cell respiration accompanied by a reversible inhibition of cell division. Other workers (3) have observed an inhibition of the Pasteur effect in tumor tissue by substituted phenols.

Cell division of sea urchins' eggs has long been known to require the presence of oxygen (4,5). A sufficiently high concentration of potassium cyanide or phenylurethane (6) likewise will inhibit cell division. These relationships have lead Krah1 and others to investigate the specific inhibition of the respiratory system by the substituted phenols that leads to the blocking of cell division (7).

Krah1 has found no apparent effect of physiologically active substituted phenols such as 4,6-dinitro-o-cresol, on metal containing oxidative systems. He also detected no effect on a number of dehydrogenases (1). However, Krah1 has been able to inhibit the action of two flavo-proteids, d-amino-acid oxidase and heartmuscle flavo-protein. Among the substituted phenols employed were 4,6-dinitro-o-cresol, 2,4-dinitro-o-cyclohexyl phenol and 2,4,5-trichloro phenol.

A study of the effect of substituted phenols on the cytochrome c reductase, a typical flavo-proteid, cytochrome c-cytochrome c oxidase system has been initiated in this laboratory.

The purpose of the investigation reported on here is to establish the effect of 2,4-dinitro-o-cyclo-hexyl phenol on the cytochrome c oxidase portion of the system.

In 1937, Keilin suggested that the enzyme known as indophenol oxidase should more correctly be termed cytochrome oxidase. On the other hand, the enzyme that was specific for the oxidation of cytochrome could not be specified by the term indophenol oxidase. Cytochrome c oxidase is now conceived of as a complex of metal bearing catalysts that oxidize reduced cytochrome c and are reoxidized by oxygen. Warburg's "Sauerstoffübertragende Ferment" (8) unquestionably plays an important role in the complex.

Cytochrome c oxidase has not as yet been obtained in solution and all work conducted with it has been done with suspensions of tissue particles to which the oxidase is associated. The difficulty of obtaining the enzyme in solution has led investigators to believe that the oxidase is associated with the cell wall and cannot be freed therefrom and retain enzymatic properties. As yet this view has been neither proven nor disproven.

OXIDASE TEST

A satisfactory test for the presence and the concentration of cytochrome c oxidase is that of Stotz and Hastings (9). In this test cytochrome c is reduced by hydroquinone and oxidized by cytochrome c oxidase. The oxidase then is itself oxidized by atmospheric oxygen. The oxygen consumed by the system is measured in a Warburg differential micro-manometer (10). In the presence of an excess of hydroquinone and cytochrome, the rate of oxygen consumption is found to be proportional to the concentration of the oxidase with a qualification to be considered below.

The conditions for saturation with hydroquinone and cytochrome c have been established by Stotz and Hastings (9).

Saturation with hydroquinone was obtained at a concentration of 1×10^{-2} molar. The solution was buffered at pH 7.15 with 0.1 M PO_4 . In this test, hydroquinone itself in the absence of both cytochrome c and the oxidase will consume oxygen.

The concentration of the oxidase is proportional to the increase in oxygen consumption over that of the hydroquinone alone. In the absence of cytochrome c, no increase in oxygen consumption of the hydroquinone is effected by the addition of oxidase. Similarly, cytochrome c in the absence of oxidase produces no increase in the oxygen consumption of the hydroquinone. In the presence of both cytochrome c and oxidase but with no hydroquinone no oxygen is consumed. Under the conditions described, the oxygen consumption due to the presence of the oxidase can be determined by subtracting the oxygen consumption of the hydroquinone alone.

Preliminary experiments indicated that although saturation with cytochrome c was not obtained even with a concentration of 4×10^{-4} molar, at this concentration the corrected oxygen consumption was not linear to the cytochrome c concentration but was linear to the oxidase concentration. The concentration of hydroquinone in the experiments to be described was 1.3×10^{-2} molar.

PREPARATION OF MATERIALS

The cytochrome c was prepared according to the directions of Keilin and Hartree (11). A slight modification was made in the final stages of the preparation but essentially the process is that as published.

Cytochrome c oxidase may be prepared quite readily in a crude state from the directions of Stotz and Hastings (9). The acetate buffer precipitations used for the removal of cytochrome c have been described by Stotz, Sidwell and Hogness (12). The combined processes are described below.

100 gm. of fat and fascia free beef heart are twice ground in a meat grinder. The tissue is placed in a large piece of muslin and put in a 2-liter evaporating dish half filled with water at 40-45° C. It is washed and pressed occasionally by hand for ten minutes. Wash water and juice are discarded and the operation repeated with cold water. A total of four hot and four cold washings are performed. The pressed mass is ground thoroughly with an equal volume of sand and 200 cc. of M/15 K_2HPO_4 . After the mixture becomes a light brown homogeneous paste, it is allowed to stand for thirty minutes with occasional stirring. The mixture is then centrifuged for twenty minutes and filtered through muslin.

The filtered phosphate suspension is treated with an equal volume of 0.2 M acetate buffer, pH 4.5. The precipitate is removed on the centrifuge and resuspended in 0.1 M PO_4 buffer, pH 7.4. The treatment is repeated once. Solution A.

EXPERIMENTAL

To study the effect of the substituted phenol, 2,4-dinitro-o-cyclohexyl phenol, on the oxidase system, it was necessary to establish the following facts:

1. The system was oxidizing hydroquinone.
2. The system was operating through cytochrome c.
3. The system was operating through cytochrome c oxidase.
4. The corrected oxygen consumption was proportional to the oxidase concentration.

Of the following experiments, Experiment I demonstrates the dependence of the system on hydroquinone and cytochrome c. Experiment II demonstrates the functioning of cytochrome c oxidase by cyanide inhibition. Experiment III demonstrates the linear relationship of the corrected oxygen consumption and the oxidase

concentration. Experiment IV demonstrates the effect of 2,4-dinitro-o-cyclohexyl-phenol on the oxidase system.

Solutions and Reagents

Buffer: 0.5 M PO_4 , pH 7.15

Hydroquinone: 18.2 mg/cc.; 0.20 cc. = 0.033 mM

Alkali: 2 M KOH

Cytochrome c: 9×10^{-7} moles/cc.; 0.15 cc. = 1.3×10^{-4} mM

Oxidase: Solution A; HI = Solution A heated at 85°C . for 15 minutes.

Cyanide: 6×10^{-7} moles KCN/cc. in 0.1 M PO_4 , pH 7.15

2,4-dinitro-o-cyclohexyl phenol: 0.005 mM/cc.

Experiment I. Dependence of Reaction on
Hydroquinone and Cytochrome C

T = 25°C .	Vessel			
Reagent	I	II	III	IV
Main Chamber				
Buffer	0.125 cc.	0.125 cc.	0.125 cc.	0.125 cc.
Water	1.425	1.625	1.875	1.425
Cytochrome c	0.45	0.45	--	0.45
Oxidase	0.20	0.20	0.20	0.20 HI
Side Arm				
Hydroquinone	0.20	--	0.20	0.20
Center Cup				
Alkali	0.10	0.10	0.10	0.10
Oxygen consumption (cmm/hr.)	316	4	10	50
Net oxygen consumption (cmm/hr.)	266			

Experiment II. Cyanide Inhibition
of Reaction

T = 25° C.	Vessel			
Reagent	I	II	III	IV
Main Chamber				
Buffer	0.125 cc.	0.125 cc.	0.045 cc.	0.045 cc.
Water	1.575	1.575	1.255	1.255
Cytochrome c	0.45	0.45	0.45	0.45
Oxidase	0.05	0.05 HI	0.05	0.05 HI
Cyanide	--	--	0.40	0.40
Side Arm				
Hydroquinone	0.20	0.20	0.20	0.20
Center Cup				
Alkali	0.10	0.10	0.10	0.10
Oxygen consumption (cmm/hr.)	136	30	38	30
Net oxygen consumption (cmm/hr.)	106		8	

Per cent inhibition:

92.5%

Experiment III. Relationship of Corrected Oxygen
Consumption to Oxidase Concentration

T = 25° C.	Vessel				
Reagent	I	II	III	IV	V
Main Chamber					
Buffer	0.125cc.	0.125cc.	0.125cc.	0.125cc.	0.125cc.
Water	1.575	1.525	1.425	1.225	1.425
Cytochrome c	0.45	0.45	0.45	0.45	0.45
Oxidase	0.05	0.10	0.20	0.40	0.20 HI
Side Arm					
Hydroquinone	0.20	0.20	0.20	0.20	0.20
Center Cup					
Alkali	0.10	0.10	0.10	0.10	0.10
Oxygen consumption (cmm/hr.)	66	97	151	253	32
Net oxygen consumption (cmm/hr.)	34	65	119	221	

Plot of net oxygen consumption vs. concentration of oxidase in
Fig. 4.

Experiment IV. Effect of 2,4-dinitro-o-cyclohexyl Phenol
on the Oxygen Consumption of the Oxidase System

T = 25° C.	Vessel				
Reagent	I	II	III	IV	V
Main Chamber					
Buffer	0.125cc.	0.125cc.	0.125cc.	0.125cc.	0.125cc.
Water	1.525	1.525	1.025	1.025	1.125
Cytochrome c	0.45	0.45	0.45	0.45	0.45
Oxidase	0.15	0.15 HI	0.15	0.15 HI	0.15
2,4-D.C.H.P.	--	--	0.50	0.50	0.50
Side Arm					
Hydroquinone	0.20	0.20	0.20	0.20	--
Center Cup					
Alkali	0.10	0.10	0.10	0.10	0.10
Oxygen consumption (cmm/hr.)	198	52	214	54	4
Net oxygen consumption (cmm/hr.)	146		160		

Per cent increase in oxygen consumption:

9.5%

DISCUSSION AND CONCLUSION

The oxidation of hydroquinone in the absence of a complete enzyme system is apparently effected by heavy metal catalysis. To maintain the concentration of heavy metal ions at the same level both in the presence and in the absence of the oxidase, heat inactivated oxidase is added to the control. To a small degree the cytochrome c stimulates the oxidation of the hydroquinone in the presence of heat inactivated oxidase as in Experiment I. This effect is a constant factor, however, and may be neglected.

Experiments I, II and III demonstrate well that the system consuming oxygen is the cytochrome c-cytochrome c oxidase

system. The functioning of the hydroquinone as substrate, the necessity for the presence of cytochrome c, the inhibition by cyanide and the linear relationship between the corrected oxygen consumption and the oxidase concentration are accepted criteria for the presence of the system.

Addition of 2,4-dinitro-o-cyclohexyl phenol to the system in Experiment IV produced a slight increase in oxygen uptake. That this was not due to the oxidation of the substituted phenol is evident by the absence of oxygen consumption when the substituted phenol but not hydroquinone was present. Under the conditions of the experiments, a linear relationship between corrected oxygen uptake and oxidase concentration, an inhibition of the oxidase would have been readily detected.

From these experiments it may be concluded that a concentration of 10^{-3} M 2,4-dinitro-o-cyclohexyl phenol does not inhibit the heavy metal bearing cytochrome c oxidase system. On the other hand a slight acceleration detected in the oxygen consumption at such concentrations of the substituted phenol is apparently not due to the oxidation of the added compound.

SUMMARY

1. No inhibition was found when 2,4-dinitro-o-cyclohexyl phenol was added to a respiring cytochrome c oxidase system.
2. A slight increase in respiration was noted on the addition of the substituted phenol.

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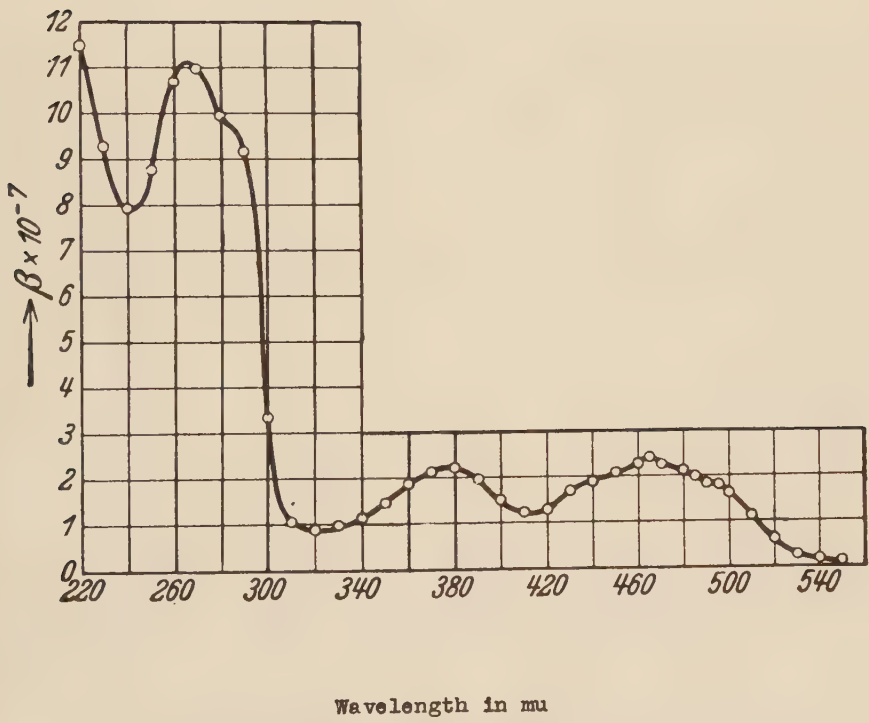


Fig. 1.--Absorption spectrum of "old" yellow enzyme

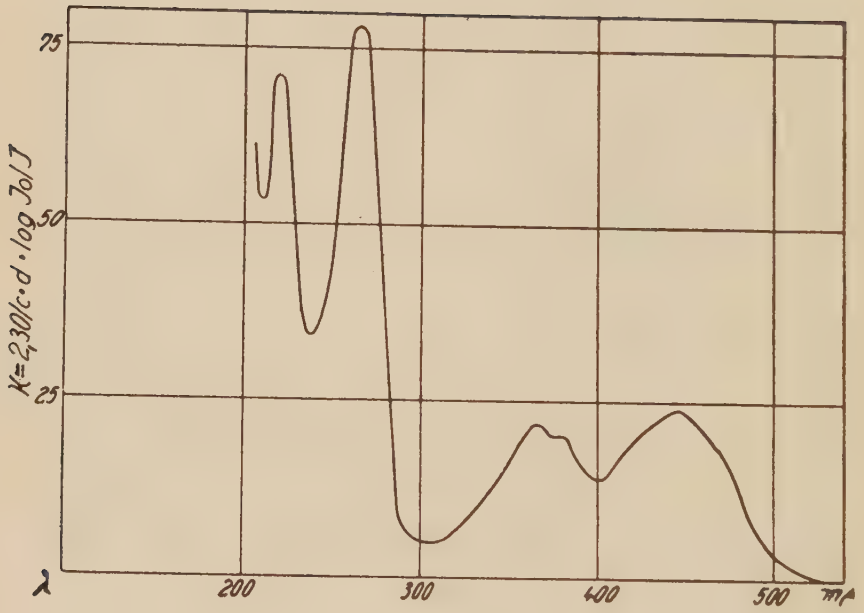


Fig. 2.--Absorption spectrum of lactoflavine

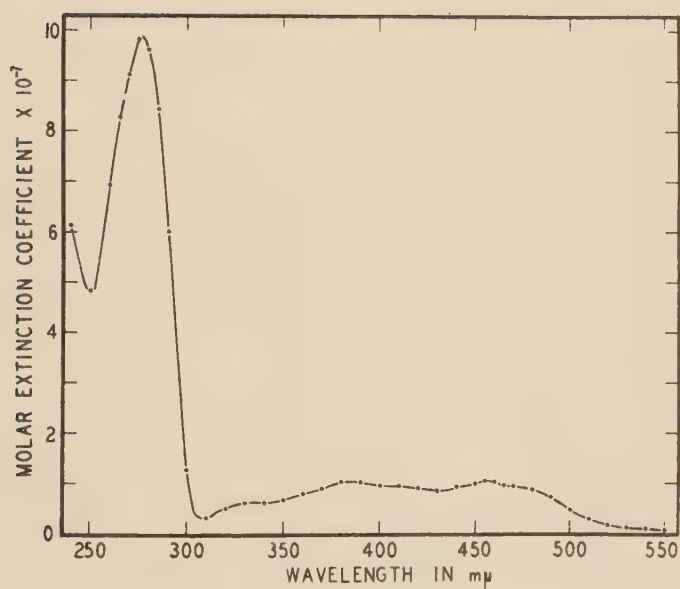


Fig. 3.--Absorption spectrum cytochrome c reductase

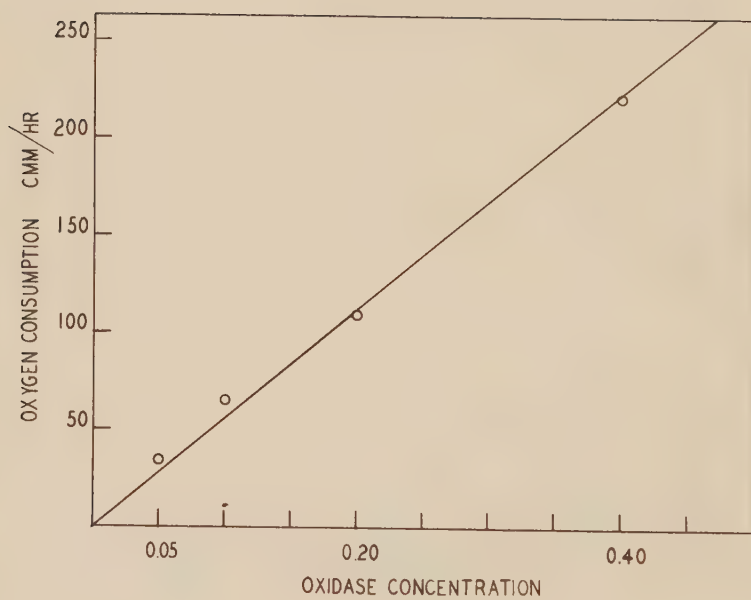


Fig. 4

